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Determination of Tissue Iron and Ferritin in Liver Pathology Comparison of Histochemical and Biochemical Results

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Summary: Results are presented from the determination of tissue iron and ferritin in 15 pathological livers (6 with steatosis, 8 with fibrosis/cirrhosis and 1 with haemochromatosis). The histological assessment according to the *Rowe* system, after *Perls'* staining, was compared with the measurement of the iron content in liver homogenate by flameless atomic absorption spectrophotometry.

The results of the flameless atomic absorption spectrophotometry were less accurate than in normal livers, but the method can still be considered reliable with satisfactory precision.

As in normal livers, the range of chemically determined values in the histological staining grades was considerable and there was quite an overlap between consecutive grades. The chemical determination of liver iron content is to be preferred.

The levels of ferritin protein and ferritin iron follow the same pattern as the total iron content.

Introduction

The liver iron content is used as the parameter for the determination of iron overload, e. g. in haemochromatosis.

Although the liver is generally considered as a homogeneous tissue, this is not always the case, especially in pathological conditions (1).

Histological assessment of the quantity of stainable iron, using systems like those of *Rowe, Scheuer, Brissot,* and other authors, is inaccurate and unsatisfactory. Chemical analysis is more reproducible, although some chemical methods have quite a large coefficient of variation. This may be due to an inhomogeneous distribution of iron, especially in cirrhosis (2). It is thus not always clear how the results of one biopsy or consecutive biopsies from patients should be interpreted. Other authors report that the iron content does not differ in needle biopsy specimens taken from the left, right or central lobes of the liver (3). It is presumed that these conflicting data might be method-dependent when the influence of blood contamination is excluded (4).

Recently we suggested a refinement of tissue iron determination by flameless atomic absorption spectrophotometry, using a liver homogenate, and expressing the amount of iron in µg per mg protein. In this way some steps in the procedure can be bypassed, and inherent inaccuracies can be avoided. In 60 specimens of grossly normal liver tissue we found a CV duplo of 8% within one specimen, and of 14% between specimens from different sites of one liver (5).

In this paper we present the results of our method, applied to specimens of livers with steatosis, or fibrosis/cirrhosis.

Materials and Methods

Materials

Liver tissue and sample preparation

Fresh human liver tissue was obtained from 15 autopsies with macroscopically abnormal livers: six with steatosis, eight with fibrosis/cirrhosis and one liver from a patient with haemochromatosis. From each liver two samples were taken, one from the left lobe and one from the right lobe. One part of each sample was put in 40 g/l formaldehyde for fixation and processed for routine histological examination, the other part was cut into small pieces (± 10 mg), frozen in liquid nitrogen and stored at −80 °C until chemical analysis. Thawed material was washed three times with 15 ml ice cold isotonic saline for 5 min. Liver was then homogenized (200 µl H_2O per 10 mg washed liver tissue), using 20 strokes of a loosely fitting pestle in a glass homogenizer.

Methods

Histological examination

The liver specimens were stained with haematoxylin-eosin (HE), Prussian blue (*Perls*' stain), *Van Gieson*'s stain, anti-ferritin antibodies and a combined *Perls*' and anti-ferritin antibodies stain.

For anti-ferritin staining the following method was used: deparaffination by means of xylol-alcohol 96% (ratio 2+1), washing for 10 min in methanol-H_2O_2 (5 g/l), washing with distilled water, washing with Tris buffer, pre-incubation with 10 g/l bovine serum albumin in Tris buffer pH 7.6, 0.05 mol/l for 15 min, incubation with rabbit-antihuman ferritin (Dako, Dakopatts a/s, Kopenhagen) 1:100 for 1 hour, washing with Tris buffer, incubation with swine-antirabbit IgG fixed to peroxidase (Dako) 1:60, for ½ hour, washing with Tris buffer, incubation in diaminobenzidine tetrahydrochloride (7 g/l 0.05 mol/l Tris, pH 7.6) (Sigma, St. Louis, USA) for 5 min, washing with water, nuclear staining with haematoxylin for 5 min, washing with water, dehydration and covering.

The grade of iron staining was assessed visually by microscopy using the modified *Rowe* system for determination of the iron content in the *Perls*' stain (6). The *Brissot* system (7) was used to ascertain the grade of anti-ferritin staining. This system was chosen on account of the inclusion of *Kupffer* cells in the calculation of the final grade of staining.

Chemical examination

1. Liver iron determination

Flameless atomic absorption spectrophotometry was performed on a Perkin Elmer (Überlingen, F. R. G.) model 3030 spectrophotometer with a deuterium background correction, equipped with a PE HGA 500 graphite furnace atomizer (programmable) and a PE AG 40 autosampler. The iron hollow cathode lamp was from Instrumentation Laboratory, no. 89225 (Paderno, Italy). The samples were diluted at least 200 times with 200 mmol/l ammonium acetate containing 1.2 g/l nitric acid, before analysis. The measurement was performed according to *Kreeftenberg* et al. (2) with some modifications. Calibration mixtures were prepared from a ferric nitrate standard solution (178 µmol/l) in 200 mmol/l ammonium acetate containing 1.2 g/l nitric acid. A linear calibration was obtained for an iron concentration range of 0, 0.53, 1.07 and 1.61 µmol/l. The accuracy of the method was monitored with acid-digested liver homogenate, stored at −80 °C in polypropylene cups.

The measurement of the protein concentration was performed according to *Lowry* et al. (8).

2. Ferritin determination

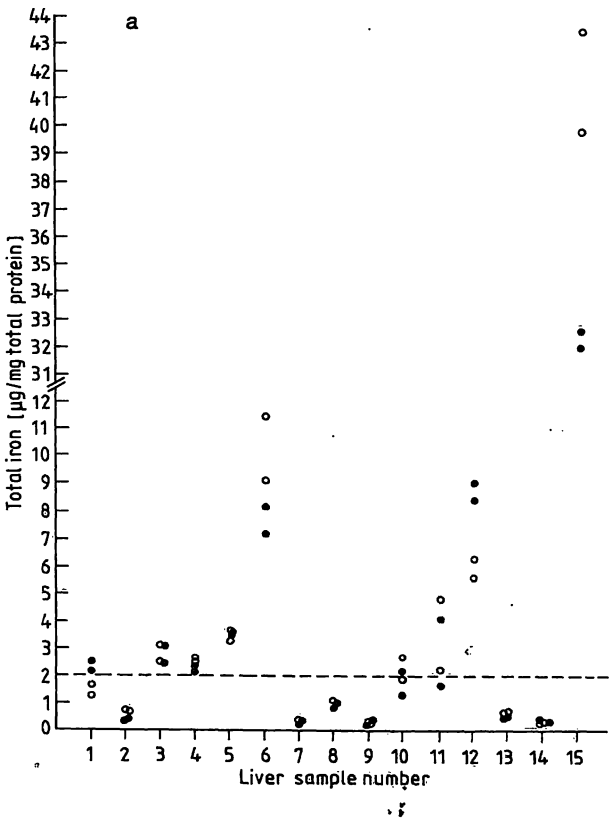
Liver ferritin was isolated from homogenates (ca. 4 g/l protein) by heat treatment (70−75 °C, 5 min) and centrifugation was performed as described by *Zuyderhoudt* et al. (4). Ferritin iron was measured in the supernatant, diluted (1+2) with 200 mmol/l ammonium acetate, 30 mmol/l nitric acid. Ferritin protein was measured by two site solid phase immunoenzymetric assay (Hibritech Europe, Liège, Belgium) according to the manufacturer's instructions.

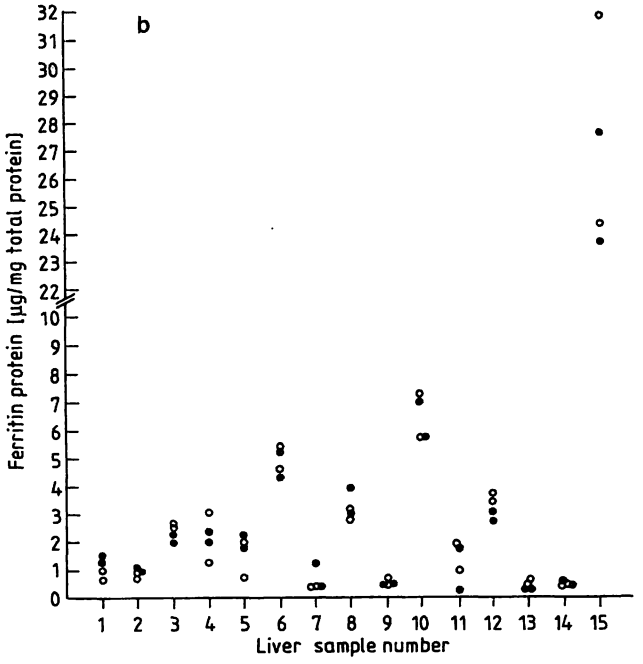
Ferritin iron determination: after preparing the homogenate as described above, the amount of ferritin iron was determined in the supernatant by flameless atomic absorption spectrophotometry. The result was expressed as µg Fe per mg protein.

Each sample was analysed in duplicate.

Results

In figure 1, the results of the analysis of the total iron content, the ferritin protein and the ferritin iron in 15 livers are presented for each liver, giving duplicate





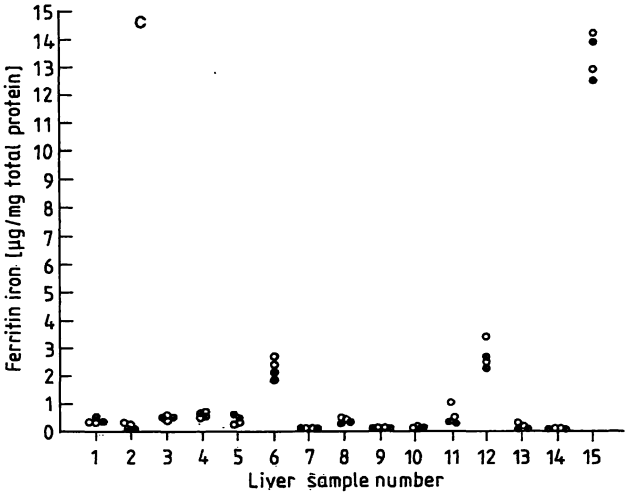


Fig. 1. Total iron (fig. 1a), ferritin protein (fig. 1b) and ferritin iron (fig. 1c) in 15 pathological liver tissue specimens.
o right lobe of the liver (duplicate samples)
• left lobe of the liver (duplicate samples)
Specimens 1–6: liver specimens with steatosis;
specimens 7–14: liver specimens with fibrosis/cirrhosis;
specimen 15: liver specimen with haemochromatosis.

Tab. 1. Total liver iron content, ferritin protein, and ferritin iron in steatotic and fibrotic/cirrhotic livers: mean, standard deviation, and range.

	Mean ± SD	range
Total liver iron (µg/mg protein)		
Steatosis (N = 6)	3.16 ± 2.45	0.36 – 7.68
Fibrosis/cirrhosis (N = 8)	2.02 ± 2.87	0.3 – 8.7
Ferritin protein (µg/mg protein)		
Steatosis (N = 6)	2.25 ± 1.3	0.98 – 4.75
Fibrosis/cirrhosis (N = 8)	1.22 ± 1.22	0.32 – 3.44
Ferritin iron (µg/mg protein)		
Steatosis (N = 6)	0.69 ± 0.64	0.13 – 1.96
Fibrosis/cirrhosis (N = 8)	0.54 ± 0.82	0.05 – 2.48

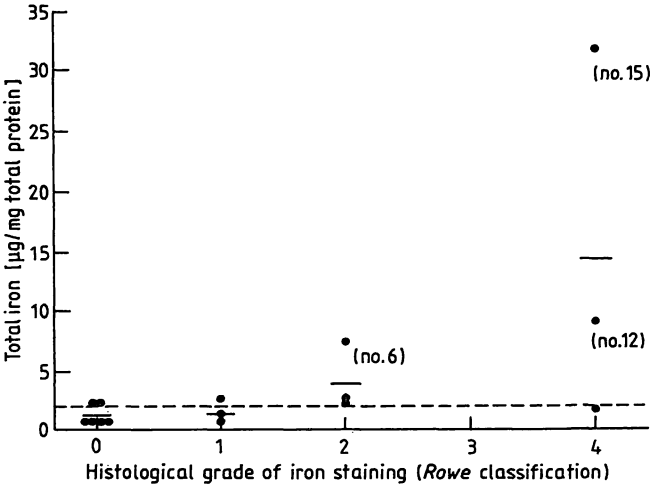


Fig. 2. Histological grade of iron staining (*Rowe* classification) vs. chemical iron determination (see text) of 15 pathological liver specimens.
Abnormal values: liver specimen No. 6 (steatosis), No. 12 (cirrhosis) and No. 15 (haemochromatosis).

values for both the left and right lobes, for all parameters. The mean value, standard deviation and range of liver iron content, ferritin protein and ferritin iron were calculated for the steatotic livers and the livers with fibrosis/cirrhosis separately and these are presented in table 1.

The relation between the degree of iron staining according to the modified *Rowe* system and the total iron content is presented graphically in figure 2. The grade of histological anti-ferritin staining according to the *Brissot* system and the total iron content determined by means of the flameless atomic absorption spectrophotometry is presented graphically in figure 3. The relation between the histological grade of anti-ferritin stain and the biochemical determination of the liver ferritin protein content is visualized graphically in figure 4.

Discussion

The determination of the liver iron content by means of flameless atomic absorption spectrophotometry after homogenisation of the specimen has proved to be an accurate procedure in normal liver tissue (5).

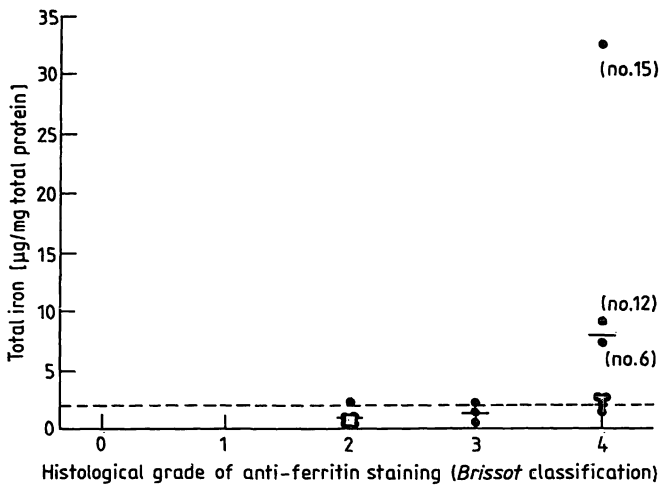


Fig. 3. Histological grade of anti-ferritin staining (*Brissot* classification) vs. chemical iron determination (see text) of 15 pathological liver specimens.
Abnormal values: liver specimen No. 6 (steatosis), No. 12 (cirrhosis) and No. 15 (haemochromatosis).

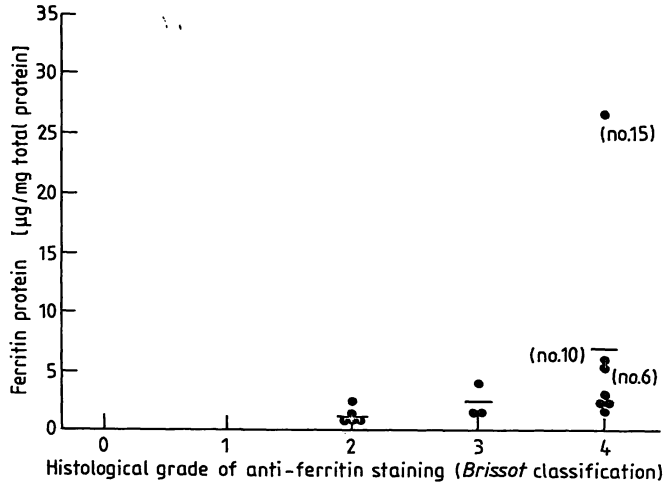


Fig. 4. Histological grade of anti-ferritin staining (*Brissot* classification) vs. immunochemical ferritin determination (see text) in 15 pathological liver specimens.

In this paper the result of this same method, applied to abnormal liver tissue is presented. The material consisted of 6 livers with steatosis and 9 livers with fibrosis or cirrhosis, one of which had haemochromatosis.

Figure 2 illustrates the wide range and overlap of the results of the histochemical estimation of the stainable iron, compared with the chemical determination of the liver iron content. This has been shown previously for normal livers and it also holds true for abnormal livers.

It is seen (fig. 1a) that the values determined in one liver specimen correspond quite well. Duplicate values within one liver lobe are most acceptable, possibly with the exception of one steatotic liver (specimen No. 6) and the strongly elevated values as found in the haemochromatosis liver (specimen No. 15). Although the values in the haemochromatotic liver in the right lobe are different, values in the left lobe are very close to each other; the overall coefficient of variation is only 15.2%. On the whole, the method for iron determination can be regarded as useful and reliable, also in livers with steatosis and fibrosis/cirrhosis, although the reproducibility is slightly less than in normal liver tissue. From table 1 it can be concluded that there are quite considerable differences in total liver iron content between livers, both in the steatosis and especially in the fibrosis/cirrhosis group. As was to be expected, the liver from the haemochromatosis patient contained an excessively high amount of iron.

The results of the liver ferritin protein determination are presented in figure 1b. From figure 1c it is apparent that the values for ferritin iron in this series of pathological livers are remarkably constant, again with the exception of steatotic liver No. 6, cirrhosis liver specimen No. 12 and, as expected, the haemochromatosis liver specimen No. 15. Mean value, standard deviation and range are given in table 1.

Figure 3 shows the relationship between the grade of antiferritin staining of the liver and the liver iron content. The number of specimens is small, but it can be observed, that with higher grades of anti-ferritin staining the liver iron content increases.

Figure 4 illustrates the relationship between the grade of anti-ferritin staining of the liver and the biochemically determined ferritin content. With higher grades of anti-ferritin staining, the biochemically determined ferritin content increases. As with the iron measurements, however, the range within a grade is quite large, and there is an overlap between consecutive grades.

In individual slides, stained with anti-ferritin antibodies and counterstained with *Perls*' blue, it was striking that within cells there was frequently no correspondence between haemosiderin content and ferritin content.

Conclusions

In conclusion, the chemical determination of liver iron is indicated in steatotic and fibrotic/cirrhotic livers as well as in normal livers, in case information about the iron content is requested. The flameless atomic

absorption spectrophotometry of liver homogenate is less accurate in these abnormal livers, but can still be considered a reliable method with satisfactory precision. Combination of chemical determination with histological/histochemical examination of liver specimens is still necessary for the diagnosis of liver disease, and for the assessment of the degree of inhomogeneity of liver tissue.

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Erratum

The regression equation of figure 1 in one of the preceding papers by C. van Deursen et al., this j. *26*, 617–622, 1988, should read: $y = 1.86\,x - 0.21$.